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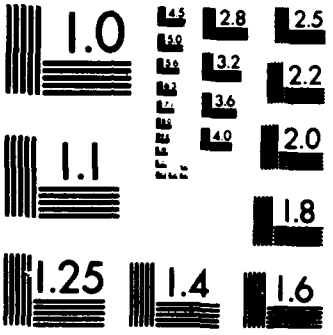

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The research objectives of this project are to characterize a marine methanotroph, develop genetic systems for this organism, and use these techniques to clone and characterize key C-1 genes. We have studied a fast-growing marine methanotroph isolated in our laboratory, *Methylomonas* sp. strain A4. We have characterized this strain in general terms, and have purified and characterized the methanol oxidase system also. Using a heterologous gene probe and mutant complementation, we have isolated and characterized two genes of the methanol oxidase system, *mox*F and *mox*A3. These genes will now be used to carry out an extensive study of promoters and gene expression in this methanotroph.

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Pasadena, CA 91125

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FINAL REPORT

Genetics in Marine Methane-oxidizing Bacteria

Mary E. Lidstrom, PI
September 1, 1984 - August 31, 1987

I. Project Goals

The goals of this project were to

a) characterize Methylomonas A4, microbiologically and biochemically
b) develop a gene transfer system for Methylomonas A4
c) isolate and map genes necessary for growth on one-carbon compounds in this strain
d) study transcriptional regulation of these genes

II. Summary of Accomplishments

We have carried out the first three goals, and have begun the final goal this summer. We have also collaborated with Colleen Cavanaugh to study the methanotrophic symbiosis in seep mussels and with Jon Sieburth to help characterize his new marine methanotrophs.

A. Characterization of Marine *Methylomonas* Strains

Methylomonas A4 was isolated from enrichments of samples from the Hyperion sewage outfall near Los Angeles, CA. We have characterized this strain, and have submitted a manuscript describing its general properties. A4 is a gram-negative motile rod with a temperature optimum of 37C and an absolute NaCl requirement. It grows well over a NaCl range of 0.5-1.5%. It contains Type I membranes and has the typical Type I %G+C ratio (55%) but containes enzymes of both the serine and ribulose monophosphate pathways for formaldehyde assimilation. This is not unprecedented in Type I methanotrophs, but it is unusual. A4 has a minimum generation time in batch culture of 3.5 hr and produces defined colonies on agar plates in 2-3 days. This is in contrast to most strains of methanotrophs which show much slower growth, especially on agar plates. A4 grows well on methanol but not on any tested substrates containing C-C bonds.

We have also been involved in the characterization of other marine Methylomonas strains, isolated by J. Sieburth. These were isolated from enrichments of samples from the Sargasso Sea, and represent much more typical Type I Methylomonas strains. They do not have the rapid growth properties of Methylomonas A4. A paper describing this work has been published.

B. Biochemical Characterization of C-1 Functions

In order to obtain biochemical data for the gene cloning experiments, we have purified and characterized the methanol oxidase system from the marine methanotroph, Methylomonas A4. This work has been submitted for publication. This includes the methanol dehydrogenase and five cytochromes, cytochrome $\underline{c}_{554}$H, $\underline{c}_{554}$L, $\underline{c}_{553}$, $\underline{c}_{552}$ and $\underline{c}_{551}$. Cytochromes $\underline{c}_{554}$ H and L may be forms of the same cytochrome. Together, they represent the major cytochrome c species in the cell, and preliminary evidence suggests they may be the immediate electron acceptor for the methanol dehydrogenase. The methanol oxidase system has general similarities to that found in other methylotrophs, but it differs in some characteristics including pI, molecular weight, and spectral properties of the components. Cytochrome $\underline{c}_{551}$ is a diheme, and is an interesting candidate for the electron acceptor of the particulate methane mono-oxygenase. Future experiments will address this possibility. We have antisera against the methanol dehydrogenase and are currently making antisera against all four of the cytochromes. We will use this antibody for dual gold label localization studies which may provide some hints for cytochrome function. In addition, the antisera will be used for screening putative clones and potential mutants for each of the cytochromes.

C. Development of Genetic Systems

We have been successful in using the IncP mobilization vector systems that we have used for genetics in other methylotrophs with A4. These vectors are mobilized into A4 from E. coli via three-way filter matings at a frequency of approximately 10^{-2} per recipient. We have shown that the broad-host range promoter cloning vehicle pDA4626 is mobilizable into A4 and that lacz is expressed and can be detected using Xgal plates. We have carried out mutagenesis experiments and have shown that EMS, NTG and UV light do not noticeably increase the spontaneous mutation frequency to drug resistances, even at mutagen dosages resulting in 0.1% survival. This is in accord with similar experiments carried out with other methanotrophs.

D. Isolation and Characterization of C-1 Genes

Two approaches have been used to isolate C-1 specific genes from Methylomonas A4. The first has involved complementation of mutants defective in methanol oxidation (Mox mutants) in the facultative methanol utilizer, Methylobacterium AM1. Under DOE support, we have extensively characterized the genetics of the Mox system in this organism, and so we have a suite of mutants and gene probes available. We have constructed a genomic cosmid clone bank of Methylomonas A4 DNA (HindIII partials) using an IncP broad-host range vector derived from pVK100, for complementing the Methylobacterium AM1 Mox mutants. One of these mutants (Mox A3) defective in cofactor-apoprotein association in the methanol dehydrogenase has been complemented. None of the other nine mutant classes were complemented by the Methylomonas A4 clone bank. We are now characterizing the complementing clone, which contains a 7.8 kb HindIII fragment, and the complementing region has been narrowed down to a 1.8 kb EcoRI-HindIII fragment. This fragment will be subcloned and used for Tn5 mutagenesis and promoter cloning.

The second approach has involved the use of a gene probe to detect Mox genes by DNA-DNA hybridization. An open-reading frame fragment (ORF 9) of the methanol dehydrogenase structural gene (moxF) from Methylobacterium AM1 hybridizes to two HindIII fragments of Methylomonas A4 DNA, 2.8 and 3.2 kb in size. Colony blots have been screened with this probe, and a Methylomonas A4 clone that hybridizes has been detected. This clone contains several HindIII fragments, including two at 2.8 and 3.2 kb. We are currently screening digests to confirm the hybridizing species. We will then express appropriate subclones in E. coli using the T7 expression system described in the proposal, and confirm the presence of methanol dehydrogenase protein with Western blots.

E. Studies of the Methanotrophic Symbiosis in Seep Mussels

As a side project with future relevance to our current studies, we have collaborated with Colleen Cavanaugh to study the proposed symbiosis between Type I methanotrophs and the gills of mussels found at the Florida Escarpment seeps. A paper describing this work has been published. We found bacteria-like bodies restricted to gill tissue, and were able to detect several C-1 enzymes, including those for the Type I assimilation pathway specifically in gill tissue. More recent work by Dr. Cavanaugh with fresh tissue has demonstrated methane uptake and incorporation in the mussel tissue. We are continuing this collaboration in an attempt to isolate and characterize the symbionts.

III. Summary and Plans

These experiments have resulted in the first isolation and characterization of key C-1 genes in a marine methanotroph. Further studies will provide the first information concerning gene organization and expression in these organisms. In addition, we expect the vent work to result in comparative genetic studies between free-living and symbiotic marine methanotrophs. Future experiments will concentrate on the structure and function of C-1 genes, including the definition of promoters, the presence of operons, and the sorting out of regulatory networks in the cell. These studies are crucial to the long-term commercial utilization of these bacteria, and for understanding their role in carbon cycling in the marine environment.

IV. Publications

Published:

Cavanaugh, C. M., P. R. Levering, J. S. Maki, R. Mitchell and M. E. Lidstrom. 1987. Symbiosis of methylotrophic bacteria and deep-sea mussels. Nature 325:346-348.

Sieburth, J. M., P. W. Johnson, M. A. Eberhardt, M. E. Sieracki, M. Lidstrom and D. Laux. The first methane-oxidizing bacterium from the upper mixing layer of the deep ocean: Methylomonas pelagica sp. nov. Curr. Microbiol. 14:285-293.

Submitted to Biochem. Biophys. Acta:

DiSpirito, A., J. D. Lipscomb, and M. E. Lidstrom. Cytochromes cH-554, cL-554, c-553, c-552 and c-551 from the marine methanotroph, Methylomonas sp. A4.

Submitted to Ant. v. Leew.:

Lidstrom, M. E. Isolation and characterization of marine methanotrophs.

In preparation:

DiSpirito, A., A. Hooper, and M. E. Lidstrom. Isolation and characterization of methanol dehydrogenase from the marine methanotroph, Methylomonas sp. A4, to be submitted to Biochem. J.

Major presentations:

Microbial utilization of methane and other gases
Environmental Science Gordon Conference, New Hampton, June 1986

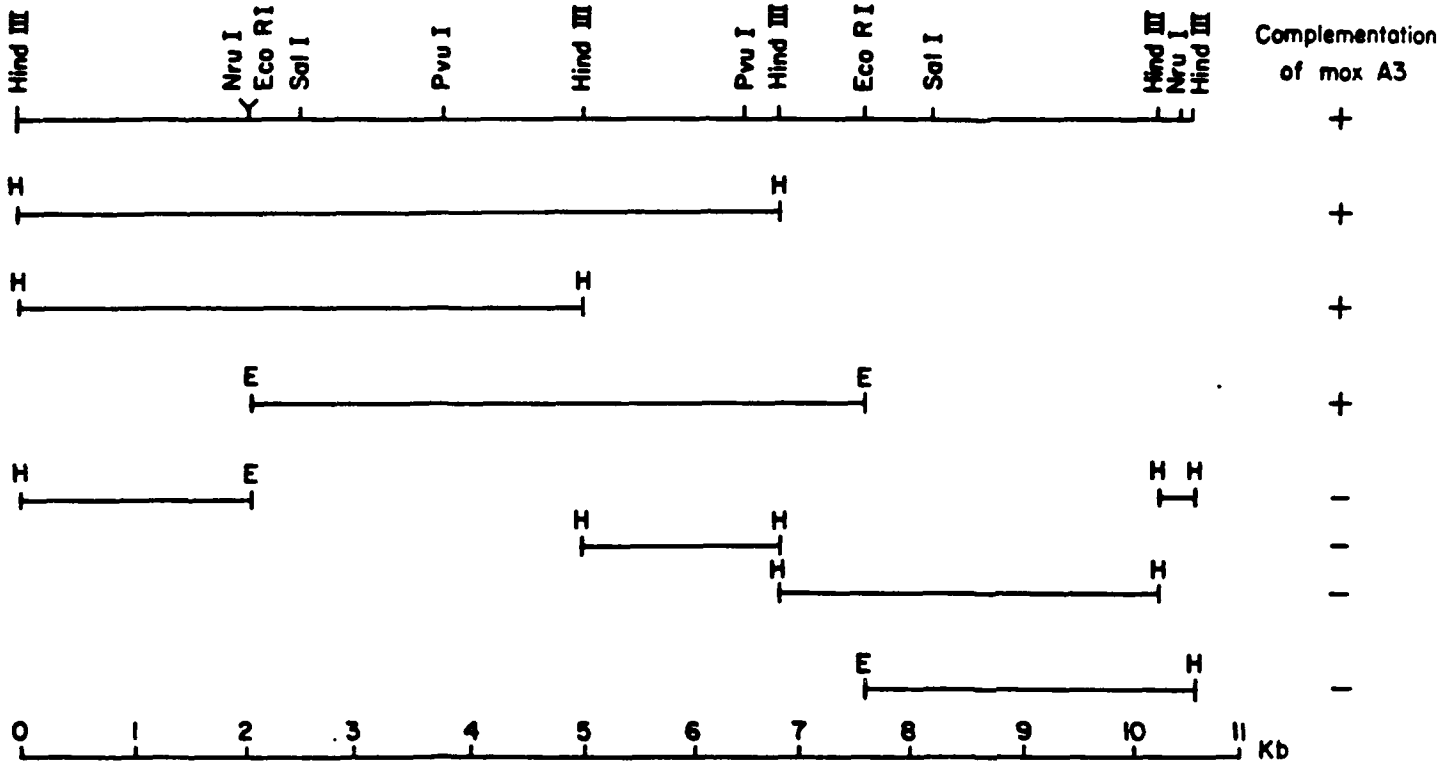
Molecular biology of methanol oxidation
5th International Meeting on C-1 Compounds, Haren, The Netherlands, August 1986

Cloning of moxF genes from Methanotrophs
Winogradsky Symposium on Chemolithotrophy, Gottingen, Germany, August 1987

V. Personnel supported:

A. DiSpirito, Post-doc	April 1985 - present
Cathy McMahan, Undergraduate	October 1984 - February 1985
Richard Stephens, Undergraduate	February 1985 - May 1985

Figure A. Complementation of the Mox A3 mutant of Methylobacterium AM1 with different subclones of Methylomonas A4 DNA, cloned into pRK310.



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